THE IDENTIFICATION OF THE PATHOGENIC ENTAMOEBA OF PANAMA*

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INTRODUCTION

The identification of the entamoeba that causes dysentery and liver abscess in Panama has recently been placed on a satisfactory basis, since two of the members of the Hospital Staff have given special attention to the subject and have had referred to them for diagnosis all cases of entamoebic dysentery. Formerly each doctor diagnosed the cases in his own ward, and there was no uniformity in the identification of entamoeba, each man determining for himself whether in a given case the entamoeba was *E. coli* or *E. histolytica.*

Influenced by the work of Schaudinn and Craig, until a year ago I regarded all pathogenic forms as *E. histolytica.* In August, 1911, a careful study of the entamoebae found in clinical cases of entamoebic dysentery and entamoebic liver abscess was begun, and observations since then have led me to be of the opinion that the only pathogenic form in this region is *E. tetragena*, Viereck.

Having ample facilities for the collection of material from clinical cases, autopsies and the operating room, and for animal experimentation, attempts were made to determine the specific characters of the entamoebae so collected.

METHODS OF EXAMINATION

These were:—

1. Fresh and moist-chamber preparations and those stained intravitam with Gentian violet.

2. Wet-fixed preparations stained with haematoxylin, Romanowsky modifications and various other stains.

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3. Dry-fixed preparations stained by modifications of the Romanowsky method.

4. Animal inoculations and feeding experiments.

EXAMINATION OF FRESH PREPARATIONS

It was found that very few of the published descriptions of entamoebae were sufficiently helpful as guides for the purposes of identification, in fact every step required to be specially worked out, and clinical and morphological data correlated with animal experimentation.

Take the questions of refractility of the ectoplasm, and the colour of the cytoplasm, it seems to me that refractility is very largely a question of the age of the entamoebae or its relation to encystment without regard to species. I have found in cultures of free living forms that the younger amoebae were not very refractile, while those approaching the period of encystment were quite refractile. In a fatal case I found that large trophozoites deep in the submucosa in the floors of ulcers were very slightly refractile, while many of the smaller entamoebae in the more superficial sloughs were highly refractile, yet they were representatives of one species. In some clinical cases, all the forms seen were highly refractile, many contained chromidia and were the precursors of the small generation, from which the cysts develop. In other cases all the entamoebae were finely granular, contained no erythrocytes and were not refractile. I have never seen either with natural or artificial illumination the green or grey tints noted by several writers, and it does not seem to me that stained specimens bear out the notion that the green colour is due to lysed erythrocytes, for the latter are held intact for a considerable period in the endoplasm and disappear by condensation and erosion, yet retaining their staining characters. I should say that all observations were made with artificial light, transmitted through blue glass, and I wish to call attention to the necessity of using some such uniform method of illumination, for the difference in illumination between light derived from a blue sky or from white clouds makes their use as sources of light uncertain, and gives inconstant pictures usually not

noted by the observer. The resulting errors may be eliminated by the use of artificial light.

The nucleus is said to be inconspicuous in the pathogenic entamoebae in fresh preparations, but I have not always found this to be the case when artificial light has been used. It is true that in many individuals, or in all the individuals in some cases, the nucleus cannot be made out in fresh preparations. But when one learns to recognise the nucleus, nothing within the trophozoite is more conspicuous. The refractile granules or masses of peripheral chromatin stand out with great prominence, and may be followed with ease as the entamoeba moves.

The presence of an ectosarc is very constant in the trophozoites of the pathogenic form, and is a very important feature for differentiation from *E. coli*.

While in most cases it is true that all entamoebae detected in stools associated with pus, blood and mucus are pathogenic, it is not this type of case that presents difficulties. The puzzling cases are those in which entamoebae are detected in fluid stools containing mucus or in semi-solid or formed stools unassociated with blood or pus, but which it is necessary to diagnose at once so that treatment may be started energetically. As an aid to rapid diagnosis, I have used with some success gentian violet as an intravitam stain. The stain is used in the concentrated fluid form or diluted with an equal amount of water, and is either added to the flake of mucus or drawn under the coverslip and the preparation examined immediately. While many individuals become over-stained or remain under-stained, usually they will gradually take up the stain in such a manner that the peripheral chromatin, the centriole and karyosome in *E. tetragena* stand out with almost the clearness and definition of well-stained haematoxylin preparations fixed in the wet way, and differentiation from *E. coli* is easy. I have tried several basic stains, but none is so good as gentian violet for intravitam staining. Even when the trophozoite is very refractory to the stain, as in the small generation, and in the forms which contain chromidia, the nucleus for some reason will become more refractile and conspicuous. The non-refractile trophozoites take up the gentian violet rapidly, while cysts and the refractile forms take up the stain slowly.

EXAMINATION OF STAINED PREPARATIONS

Haematoxylin stained preparations were made from those fresh films which contained a sufficient number of entamoebae to warrant staining and study. The coverslip was removed and both slip and slide were fixed in Schaudinn's bichloride alcohol, or in Zenker's solution diluted one-fourth and one-eighth, as the full strength solution causes artefacts in the nucleus. Several haematoxylin stains were used, and particularly clear pictures were obtained with Mallory's phosphotungstic acid haematoxylin, with the modification that the films were not given a preliminary treatment with oxalic acid or permanganate but were placed over night in the haematoxylin and then differentiated with very dilute permanganate. This is a shorter way of using phosphotungstic acid haematoxylin and gives excellent results.

Fresh and wet-fixed preparations were controlled or compared with others stained after dry fixation by some modification of the Romanowsky stain. The films were thoroughly dried then stained with Hasting's stain, over-stained with Giemsa's stain, and differentiated with ammoniated alcohol. It was observed that the descriptions of pathogenic entamoebae, as well as figures used to illustrate them in the literature, did not correspond with the results obtained in well-differentiated films stained by the method just described. And it is believed that descriptions in the literature based on films stained in the usual way, that had not been sufficiently differentiated, are not as exact as they might be. Romanowsky stains have a tendency to over-stain just as haematoxylin does, though not so intensely as the latter, and this over-staining must be corrected by the use of a differentiating agent.

It has been my practice to stain in the following way. Fresh coverslip preparations containing a sufficient number of entamoebae to warrant staining and study, or those intended for diagnosis, are made into smears by sliding off the coverslip and thoroughly drying both slip and slide, after which each is stained with Hasting's stain for 15 minutes. Satisfactory films are then over-stained with Giemsa's stain until the film has a diffuse reddish purple tint. The film is then plunged into 60% ethyl alcohol containing about 1% of water of ammonia (10%), and differentiated in this,

washed in water, and controlled by the microscope until the purple substance of the nucleus and the blue colour of the cytoplasm are strongly contrasted. The film when properly differentiated has a blue-violet colour. If the film has been greatly over-stained it is treated with a momentary douche of 95% alcohol. Beautiful pictures are obtained in this way, but what is of more importance, the various figures displayed by the purple staining substance (karyosome?) can be noted and followed with ease. This purple staining substance in the nucleus of *Entamoeba tetragena* in dried-fixed films represents only a portion of the nucleus, as the centriole and peripheral chromatin do not stain purple by the above method. The purple staining substance in the nucleus of *E. tetragena* frequently appears as a ring, or as a reticulum or scattered granules. Its phases do not appear to have been accurately described, but have been confused with the appearances presented in wet-fixed films when basic stains have been used.

Here I wish to call attention to certain errors of interpretation which I believe have resulted from the failure to properly distinguish between the purple staining substance or karyosome of the nucleus in *Entamoeba tetragena* in dry-fixed films, and the nuclear substance which stains with basic stains in wet-fixed preparations. For example, figures showing the purple chromatin of the nucleus of *E. histolytica* represented by Craig and illustrated in its proper colour, have been used by other writers and figured in terms of haematoxylin or black and white, without explaining that the black in the figure represents the purple staining substance of dry-fixed Romanowsky preparations. Dry-fixed stained films should never be confused with those stained after wet fixation, for the pictures are different in each case, and the mistake should never be made of attempting to translate one into the other. In dry-fixed films the permeability of the entamoebae to stains apparently has been profoundly modified, for the centriole and the peripheral chromatin do not take or retain the purple stain. The karyosome alone retains the purple stain, while the remainder of the nucleus stains faintly blue, sometimes revealing the achromatic granules of uniform size which appear to form part of its structure.

FEEDING AND INOCULATION EXPERIMENTS

A number of kittens, cats, dogs and monkeys have been fed by mouth, or inoculated per rectum with cysts and trophozoites of *Entamoeba tetragena.* When tetragena cysts have been fed to half-grown cats there has resulted not the typical entamoebic colitis, such as is usually described in the literature, but an enteritis, *and in this lesion in the ileum trophozoites have been found which, though arising from tetragena cysts, had the morphology not only of E. tetragena but of E. histolytica and E. nipponica.* Now as a culture, which from its history and microscopical appearance was certainly a pure culture of *E. tetragena* cysts, was used in the experiment, and as the various forms appeared in the cat's intestines, I am led to believe that the trophozoites, described as *E. histolytica* and *E. nipponica* by various writers, are nothing more than the large trophozoites in the first place and atypical or degenerate forms of *E. tetragena* in the latter. I am confirmed in this opinion by never finding in my cases any of the perpetuating forms described by Schaudinn and Craig for *E. histolytica.* In one fatal case of tetragena infection, I observed from autopsy material many trophozoites that protruded pseudopodia not unlike those figured by Hartmann and Craig for *E. histolytica.* Their extremities were refractile, and appeared to contain a round spore-like body, yet, when these coverslip preparations were fixed immediately and stained with haematoxylin and by Romanowsky, the picture presented was that of *E. tetragena*, and the spore-like bodies had disappeared. In studying a strain of *E. tetragena* in cats, following rectal injection of trophozoites, I found at the fourth remove in dry-fixed preparations a great many trophozoites, the peripheries of which contained one or several lobose projections, the interior of which were very frequently deeply stained blue and suggested strongly the descriptions of *E. histolytica* by Craig. But these were artefacts, for in wet-fixed haematoxylin preparations from the same coverslip preparation the picture was that of *E. tetragena*, and associated with these trophozoites were several uninucleate tetragena cysts. If only the dry-fixed films had been studied in this case, they might easily have been described as *E. histolytica.*

With reference to rectal inoculation of trophozoites, infection practically always follows in animals of the right age, and death occurs within a few days (five to eight) if the strain is not too aged, while by mouth feeding the duration is longer (twelve days). In this, my experiments have not paralleled those of other writers. It is difficult to explain this. I may say that after several unsuccessful attempts to infect very young kittens and adult cats, I subsequently only used kittens weighing about 700 grams.

Animal experimentation is of very great value in studying the variations in a given strain. In a strain recently I have been able to watch senility gradually developing from week to week, the entamoebae becoming reduced in size and filled with chromidia, and ultimately becoming encysted.

The comparative study of staining and fixing agents has brought out some very interesting information. The very marked differences presented by dry-fixed and wet-fixed stained preparations have been referred to. Phosphotungstic acid haematoxylin gives varying pictures depending on the fixative. More information of the structure of the nucleus is obtained by the use of this stain after fixing with diluted Zenker's fluid (1/8 and 1/4) and Flemming's fluid. The karysome is not so well transfixed when treated with Merkel's, Hermann's or Schaudinn's fluid. In wet-fixed films stained by Romanowsky it was never possible to stain the trophozoites exactly like the tissue cells near by. When stained by Giemsa's method and differentiated in acetone xylol the peripheral chromatin usually stained blue, while the centriole and nuclear sap stained pink or red, the tissue cells near by displayed purple staining substance throughout the nucleus. It was possible in several cases to study first the tissue-destroying trophozoites, later the small generation, and finally during convalescence and after apparent recovery the cysts, although in practically all cases that received energetic medication the small generation and cysts did not appear.

The detection of cysts in convalescent and recovered cases is most important, for it is the cyst, and not the trophozoite, which is the infecting agent and makes the host of the former a 'carrier.' The identification of tetragena cysts is usually easy in stained preparations and should present no difficulties in fresh films, yet

the cysts are so small (12 to 15μ in diameter) that they have frequently been mistaken for monad or coli cysts or fat droplets, or possibly mononuclear leucocytes. When in doubt and the number of cysts was to small to risk loss by fixation and staining, I have vaselined the preparation or kept it in a moist chamber, and if the cyst was homogeneous at first, after one or two days, one, two or four nuclei became distinctly visible. Tetragena cysts are more commonly detected in neglected cases which have partly recovered from entamoebic dysentery who may have diarrhoea or whose stools are solid, and I have found cysts in a case that had been insufficiently treated by means of rectal injections. It would seem, however, that if a case of dysentery is treated early and energetically the trophozoites are at once driven from the field, leaving none to develop into the drug-resistant small generation from which the cysts arise. This, it will be seen, is analogous to the rational treatment of malaria, in which the asexual generation is destroyed at once by large doses of quinine, thus destroying all the forms from which the gametes arise.

In acute or new infections, or in very active lesions, many of the trophozoites are of large size—30 to 60μ in diameter. As the strain grows older, the size often becomes reduced, the trophozoites measuring from 12 to 24μ in diameter. These forms frequently contain coarse blocks of chromidia, and they constitute the 'small generation.' During convalescence and after apparent recovery there appear small trophozoites 12 to 15μ in diameter, and associated with them are cysts and four-nucleated schizonts. If relapse occurs with symptoms of colitis, large trophozoites make their appearance again, and the cysts will have disappeared. Thus, during the progress of a case of tetragena dysentery, at first large trophozoites will be seen, many or all of them having the characters described for *E. histolytica* by Schaudinn and Craig, and later if the case has been a neglected one, the small generation with cysts will be found, of which there is no better description extant than that in Elmassian's paper on this form, which he has called *E. minuta.*

CONCLUSIONS

If there is a sufficient number of cysts they may be fed by mouth to young cats, and it may be possible to recover, from their bowel lesions, trophozoites having not only the characters of *E. tetragena*, but of *E. histolytica* and *E. nipponica* as well. Or, if in any given case of tetragena dysentery, in which the trophozoites are of the histolytica type, they be injected rectally into a young cat and the strain carried on by subsequent rectal inoculation into other cats at the time the infected animal dies, so as to prolong the vegetative phase of division, then it will be seen that the *histolytica*-like trophozoites become reduced in size, filled with chromidia, and at the fourth or fifth remove it is possible to find uninucleate tetragena cysts. The nucleus of the trophozoites meanwhile has taken on a typical tetragena appearance with a prominent karyosome. It is now impossible to infect other cats per rectum with this material. If we make dry-fixed Romanowsky stained smears of material containing these typical tetragena trophozoites, they will occasionally present the morphological peculiarities of '*E. histolytica*' described by Craig. We are thus able to correlate most of the observations of Schaudinn, Craig, Elmassian and Koidzumi, and state that there is but one pathogenic entamoeba, and that one is *E. tetragena*.

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